

## Supplementary information

### Strains and growth conditions

*Escherichia coli* DH5 $\alpha$  (Stratagene) and M15 (Qiagen), and *S. aureus* RN4220, a nitrosoguanidine-induced mutant capable of accepting *E. coli* DNA (Kreiwirth *et al*, 1983), were used for plasmid amplification and genetic manipulations. *S. aureus* strain RN6390 derives from 8325-4 and is our standard *agr*<sup>+</sup> strain. *S. aureus* WA400 ( $\Delta$ *rnaIII*) is a derivative of 8325-4 in which the P2 operon is functional but the P3 operon is deleted and replaced by the chloramphenicol transacetylase gene (*cat86*) (Janzon & Arvidson, 1990). Staphylococci were grown either on BM agar plates (1% peptone, 0.5% yeast extract, 0.1% glucose, 0.5% NaCl, 0.1% K<sub>2</sub>HPO<sub>4</sub>), or in brain-heart infusion (BHI), with erythromycin (5  $\mu$ g/ml) or kanamycin (50  $\mu$ g/ml) when appropriate.

The inactivation of the RNase III gene (*rnc*) was carried out by allelic replacement. A deletion/replacement  $\Delta$ *rnc::cat* mutant of *S. aureus* RN6390 was obtained by using pMAD, a thermo-sensitive plasmid which contains a constitutively expressed  $\beta$ -galactosidase gene thus allowing positive selection of double crossing over by following the  $\beta$ -galactosidase activity on X-gal agar plates (Arnaud *et al*, 2004). A 1036 bp DNA fragment generated by PCR using oligonucleotides cat951 and cat1987 corresponding to the chloramphenicol-acetyl-transferase gene from pC194 (Horinouchi & Weisblum, 1982) was cloned between two DNA fragments generated using oligonucleotides rna251/rna1123 (872 bp) and rna1577/rna2268 (691 bp), corresponding respectively to the chromosomal DNA regions upstream and downstream from *rnc*, in pMAD. The resulting plasmid, pLUG519, was electroporated into RN4220, and then into RN6390. Transformants were grown at the non-permissive temperature (37°C), to select for cells in which the plasmid had been integrated into the chromosome by homologous recombination. To favor the second recombination event, a single colony was grown at 30°C

for 10 generations and plated at 37°C overnight. Cells, which have lost the plasmid vector through a double crossing-over event, were detected on X-gal agar plates. PCR amplifications were used to confirm the loss of most of the *rnc* gene (codons 56 to 200), which was replaced by the *cat* gene.

### Plasmids

Total DNA and plasmid DNA were prepared with standard methods (Sambrook *et al*, 1989). Transformation of *Escherichia coli* DH5 $\alpha$  and M15 was performed by treatment with CaCl<sub>2</sub>, and *S. aureus* strains were transformed by electroporation (Bio-Rad gene pulser).

RNAIII was expressed in *Staphylococcus* by using plasmid pE194 (Horinouchi & Weisblum, 1982), which had been modified by adding an *EcoRV* restriction site next to the *XbaI* site forming pLUG274 (Benito *et al*, 2000). PCR products were digested with *HindII* and *XbaI* (site present on each primer) and ligated to the pLUG274 vector digested by *EcoRV* and *XbaI*. The ligation product was electroporated to RN4220 and the resulting plasmid was introduced into the strain WA400. The 3' domain of RNAIII was expressed under the control of the P3 promoter. The promoter sequence was amplified using primers agr-sa1819/agr-sa1569, and the 3' end of RNAIII including the transcriptional terminator (nts 394 to 514) was amplified using primers agr-sa1180/agr-sa751. The PCR products were digested by *StuI* and ligated together before being re-amplified with the external primers agr-sa1819/agr-sa751. The resulting PCR product was cloned as above on pLUG274, forming pLUG324. To express the RNAIII deleted of the whole hairpin 13 (RNAIII- $\Delta$ 2), the *agr* locus region corresponding to nts 1 to 409 of RNAIII was amplified with oligonucleotides agr-sa1819/agr-sa1163, and the region corresponding to nts 452 to 514 was amplified with primers agr-sa1119/agr-sa751, using PWO DNA polymerase (Roche). This polymerase generated blunt-ended PCR products that were ligated together and re-amplified with the external primers to

be cloned on pLUG274. The ligation product was electroporated into RN4220 and the resulting plasmid (pLUG304) was introduced into the strain WA400. To express the RNAIII deleted in loop 13 (RNAIII- $\Delta$ 1), a similar construct was done using oligonucleotides agr-sa1819/agr-sa1141 to amplify the *agr* locus region corresponding to nts 1 to 429 of RNAIII and primers agr-sa1134/agr-sa751 for the region corresponding to nts 438 to 514. The resulting plasmid was named (pLUG302).

### **Construction of transcription-translation fusions**

Plasmid pTCV-*lac*, is a low-copy-number promoter-less *lacZ* vector used for the study of promoter gene expression in Gram-positive bacteria (Poyart & Trieu-Cuot, 1997). Promoters DNA sequence were amplified from RN6390 using primers listed in table S1. PCR products were digested by *Eco*RI and *Bam*HI and ligated to plasmid pTCV-*lac* digested by the same enzymes. The ligation products were transformed in *E. coli* DH5 $\alpha$ . Selected plasmids controlled by PCR and sequenced using vlac2 primer, were then electroporated into *S. aureus* RN4220 and then to other staphylococcal backgrounds. A promoter fragment of the protein A gene (*spa*) encompassing nts -454 to +1 and nts -454 to +12 was cloned in this vector, by using DNA fragments generated by PCR with oligonucleotides spa246/spa713 and spa246/spa724, respectively. Similarly, a promoter fragment of the beta subunit of RNA polymerase gene (*rpoB*), encompassing nts -480 to +1, was generated by using oligonucleotides rpo729 /rpo1215 and cloned in this vector.

For transcriptional/translational fusions, plasmid pTCV-*lac* was modified by deletion of a region encompassing the Shine & Dalgarno sequence and the AUG sequence of *lacZ*. A *Bam*HI (site present in the multiple cloning site)-*Sac*I (site present in *lacZ*) restriction fragment of pTCV-*lac* was replaced by a DNA fragment generated by PCR using oligonucleotides vlac3 (complementary to codon n°2 of *lacZ* and containing a *Bam*HI site)

and v<sub>lac5</sub> (complementary to the sequence downstream of the *SacI* site of *lacZ*), resulting in pLUG220. A DNA fragment corresponding to *spa* mRNA (nts +1 to +63) was amplified using primers spa694/spa772, restricted by *Bgl*III (present on spa694) and ligated to the *PrpoB* DNA fragment (nts -480 to +1) digested by *Bam*HI. The ligation product was reamplified using external primers rpo729/spa772, digested by *Eco*RI and *Bam*HI and cloned onto pLUG220 digested by the same enzymes.

### **Affinity chromatography using biotinylated RNAIII**

*In vitro* transcribed RNAIII (from plasmid pLUG322) and group II intron fragment  $\Delta$ 52XBA (as RNA control) were 3' end biotinylated using biotin amidocaproyl hydrazide as previously described (Jestin *et al*, 1997). Renatured biotinylated RNA (3 nmol) were bound to 150  $\mu$ l streptavidin-agarose beads (Sigma) previously blocked by incubation at 4°C overnight in a buffer containing 50 mM triethanolamine pH 7.5, 0.2 mM EDTA, 0.5 mM DTT, 0.1 % Igepal CA-630, 0.2 mg/ml tRNA and 0.2 mg/ml BSA. After incubation of the RNAs with the beads at 4°C for 1 h in the binding buffer containing triethanolamine 50 mM pH 7.5, 100 mM KCl, 10 % DTT, 1.5 mM MgCl<sub>2</sub>, the beads were recovered by mild centrifugation and washed three times with 1 ml binding buffer. About 3-5 % of the input RNA remained bound to the beads. A *S. aureus* total extract was prepared from the strain RN6390 in the presence of RNase inhibitors and was pre-cleared by incubating 0.5 ml extract (10 mg/ml total protein concentration) with 150  $\mu$ l streptavidin agarose beads. The pre-cleared extract was then incubated (30 min at 4°C) with the streptavidin-agarose beads coupled either to the biotinylated RNAIII, to the biotinylated  $\Delta$ 52XBA (as RNA control) or without RNA. After extensive washes, proteins bound to the RNA beads were eluted by increasing NaCl concentration (from 0.1 to 1 M) in the binding buffer. After precipitation, proteins were fractionated on a 5-20 % gradient SDS-polyacrylamide gel, stained with colloidal Coomassie

Blue G250. Proteins were identified by peptide mass fingerprinting with a Voyager DE-STR matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer. The Investigator Progest system (Genomic Solutions) was used for protein digestion with modified porcine trypsin (Promega), for peptide purification, and for preparation of the MALDI plates. A minimum of four matching peptides (at 50-ppm error) and 15 % coverage of the sequence were considered necessary for positive protein identification.

### **RNase III preparation**

After cell growth, crude extract was prepared in 25 mM Tris-HCl pH 8.0, 150 mM KCl containing a protease inhibitor cocktail, and was incubated with the Ni<sup>2+</sup>-beads (Qiagen) for 1 h at 4°C. The beads were then successively washed with 25 mM Tris-HCl pH 8.0 in the presence of 400 mM KCl, and with the same buffer containing 150 mM KCl. The enzyme was eluted in the same buffer using increasing concentrations of imidazole (from 50 mM to 800 mM). After concentration and dialysis, the RNase III was kept in a storage buffer containing 50 % glycerol. Mass spectrometry analysis and N-terminal sequencing were carried out on the purified enzyme.

### **RNA preparation**

RNAIII, RNAIII derivatives ( $\Delta$ 2 deletion of nts U409 to A451, and  $\Delta$ 1 deletion of nts C430 to A437), and *spa* mRNA fragment (nts 1 to 200) were transcribed *in vitro* using T7 RNA polymerase as previously described (Benito *et al*, 2000). All RNAs carry at their 5' end two additional guanines, which have no effect on the RNA folding. *In vitro* transcribed RNAs were purified by 8 % polyacrylamide-8 M urea gel electrophoresis. After elution in 0.5 M ammonium acetate/1 mM EDTA buffer, the RNAs were precipitated twice with ethanol.

5' end-labeling of dephosphorylated RNA or DNA oligonucleotides was performed with T4 polynucleotide kinase and [ $\gamma$ - $^{32}\text{P}$ ]ATP (Sambrook *et al*, 1989), and 3' end-labeling of RNA was done with T4 RNA ligase and [ $^{32}\text{P}$ ]-pCp (England & Uhlenbeck, 1978). Labeled RNAs were purified on denaturing polyacrylamide gel electrophoresis. Before use, RNAs were renatured by incubation at 90°C for 2 min in RNase-free water, 1 min at 4°C followed by an incubation step at 20°C for 15 min in TMN buffer (20 mM Tris-acetate pH 7.5, 10 mM magnesium-acetate, 150 mM Na-acetate).

### **Determination of constants of RNAIII/spa complex formation**

Binding rate constant of RNAIII/spa complex was measured as previously described (Persson *et al*, 1988). Binding of  $^{32}\text{P}$ -labelled RNAIII to a ten fold excess of unlabelled spa was performed at 37°C in TMN buffer. Samples were withdrawn at various time points (0-1 hour), added to gel application buffer and loaded onto a native 5% polyacrylamide gel. The gel was run at 4°C and constant voltage (300 V) for 3 hours and subsequently dried. Bands corresponding to the RNAIII/spa complex and free RNAIII, respectively, were quantified using a Bio-imager Analyser BAS 2000 (Fuji).

For determination of the apparent dissociation rate constant of RNAIII/spa mRNA complex,  $^{32}\text{P}$ -labelled spa mRNA was incubated with increased molar amount of wild-type or mutant RNAIII for 15 min at 37°C in TMN buffer. Samples were then treated as described above.

### **RNA structure probing**

RNAIII-spa mRNA complex formation was carried out at 37°C for 15 min in TMN buffer with end-labeled RNA ( $3 \times 10^{-8}$  M) and a five-fold excess of the unlabeled complementary RNA. Enzymatic hydrolysis was performed in 10  $\mu\text{l}$  of TMN, in the presence of 1  $\mu\text{g}$  carrier

tRNA at 37°C for 5 min: RNase T1 (0.0025 units), RNase V1 (0.5 units), RNase T2 (0.3 units). Chemical modifications were performed on 2 pmol of *spa* mRNA at 20°C in 20  $\mu$ l of reaction buffer containing 2  $\mu$ g of carrier tRNA. Alkylation of the C(N3) and A(N1) positions was done with 1  $\mu$ l DMS (diluted 1/8 and 1/16 in ethanol) for 2 min in TMN buffer. Modification of U(N3) and G(N1) were done with 5  $\mu$ l of CMCT (50 mg/ml) for 10 and 20 min in a buffer containing 50 mM Na-borate pH 8, 5 mM MgAc, 150 mM KOAc.

End-labeled RNA fragments were sized on 12% polyacrylamide/8 M slab gels. Cleavage positions were identified by running RNase T1, and alkaline ladders of the RNA in parallel. The cleavage or modification sites of unlabeled RNAs were detected by primer extension. Details for hybridization conditions, primer extension and analysis of the data have been previously described (Benito *et al*, 2000).

## References

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